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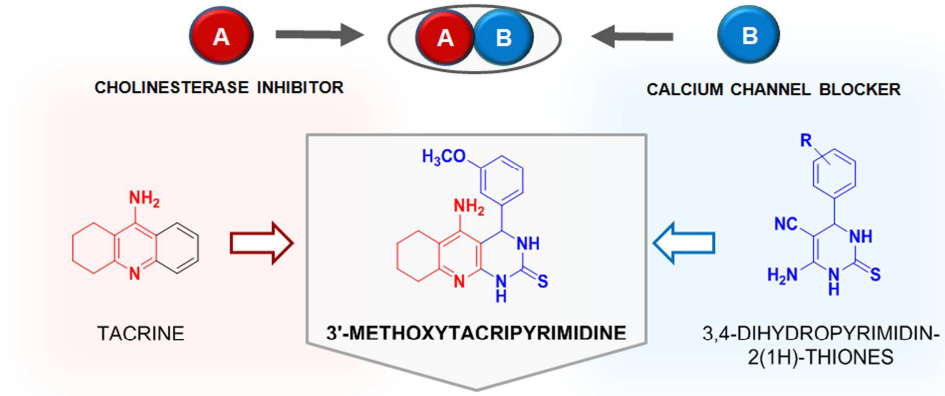

MICROMOLAR hAChE AND hBuChE INHIBITOR
MICROMOLAR CALCIUM CHANNEL BLOCKER
NO SIGNIFICANT HEPATOTOXICITY UP TO 300 µM
GOOD PREDICTED ORAL ABSORPTION AND BBB PERMEABILITY

Tacripyrimidines, the First Tacrine-Dihydropyrimidine Hybrids, as Multi-Target-Directed Ligands for Alzheimer´s Disease

Mourad Chioua,[a] Eleonora Buzzi,[a] Ignacio Moraleda,[b] Isabel Iriepa,[b] Maciej Maj,[c] Artur Wnorowski,[c] Catia Giovannini,[d] Anna Tramarin,[e] Federica Portali,[f] Lhassane Ismaili,[g] Pilar López-Alvarado,[f] Maria Laura Bolognesi,[e] Krzysztof Jóźwiak,[c] J. Carlos Menéndez,[f] José Marco-Contelles,[a,*] and Manuela Bartolini[e,*]

Abstract

Notwithstanding the combination of cholinesterase (ChE) inhibition and calcium channel blockade within a multitarget therapeutic approach is envisaged as potentially beneficial to confront Alzheimer's disease (AD), this strategy has been scarcely investigated. To explore this promising line, a series of 5-amino-4-aryl-3,4,6,7,8,9-hexahydropyrimido[4,5-b]quinoline-2(1*H*)-thiones (tacripyrimidines) (**4a**-**l**) were designed by juxtaposition of tacrine, a ChE inhibitor (ChEI), and 3,4-dihydropyrimidin-2(1*H*)-thiones, as efficient calcium channel blockers (CCBs). In agreement with their design all tacripyrimidines, except the unsubstituted parent compound and its *p*-methoxy derivative, acted as moderate to potent CCBs with activities generally similar or higher than the reference CCB drug nimodipine and were modest-to-good ChEIs. Most interestingly, the 3'-methoxy derivative (**4e**) emerged as the first well balanced ChEI/CCB agent, acting as low micromolar hChEI (3.05 µM and 3.19 µM on hAChE and hBuChE, respectively) and moderate CCB (30.4 % at 1 µM) with no significant hepatotoxicity toward HepG2 cells and good predicted oral absorption and blood brain barrier permeability.

Keywords: Alzheimer's disease; Calcium channel blockade; ChE inhibition; Molecular modeling; MultiTarget-Directed Ligands; Tacripyrimidines

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1. Introduction

Alzheimer's disease (AD) is a devastating, age-related neurodegenerative disorder [1] which has become a major and rising public health concern because of the high costs related to the management of the increasing number of AD patients, who have no effective treatment for cure. Indeed, the main class of drugs available on the market for AD treatment provides only symptomatic treatment and comprises acetylcholinesterase (AChE) inhibitors (AChEI) [2] which mitigate cognitive impairment by temporarily enhancing the cortical cholinergic tone. [3] Although their effect is mostly symptomatic, evidence from preclinical and clinical trials has suggested that cholinergic inhibitors may also exert other beneficial long-term effects by partially reducing amyloid production and its neurotoxicity [4]. In particular, the interest for AChEIs binding at the peripheral anionic binding site (PAS) of AChE has been stimulated upon the discovery of the role of this site in accelerating the aggregation of β-amyloid (Aβ). [5, 6] Since the approval of tacrine in 1993 as the first AChEI for AD treatment, several strategies have been investigated in an effort to develop disease-modifying treatments (DMTs). However, despite the potentially enormous commercial reward and the high number of drug candidates entering clinical trials, an efficient treatment is still lacking.[7] This failure has led to the recent reduction of AD-focused early discovery programs within big pharmaceutical companies, making the current and future academic contribution to this field of increasing significance.[8] Clinical failure has been partially ascribed to the complexity of this pathology, which features a multifaceted interplay of several factors, whose exact role is not yet fully understood.[7] This observation has laid down the basis for the current interest in the so-called Multitarget Directed Ligands (MTDLs), an heterogeneous class of compounds that are designed to simultaneously address more than one pathological event.[9] Based on this strategy, a number of MTDLs have been developed by modification of commercial drugs and active scaffolds.[9-17] Among those, tacrine-based multitarget derivatives have been

shown to be able to hit several key targets involved in AD and exert multiple beneficial activities both in vitro and in vivo.[14- 16] Indeed, the high ligand efficiency has made tacrine scaffold an ideal starting point for designing and achieving highly active MTDLs.[14-16]

Based on these considerations, some years ago we designed a class of MTDs that we called tacripyrines (**I**, Figure 1) by juxtaposition of tacrine and nimodipine, as reference molecules endowed with anticholinesterase and calcium antagonism profile, respectively, for the treatment of AD.[18, 19] The most interesting tacripyrine, i.e., (±)-*p*-methoxytacripyrine (*p*-MT, Figure 1), showed high selectivity toward AChE, moderate inhibition of calcium intake after potassium stimulation in SHSY5 cells, weak inhibition of the pro-aggregating action of hAChE on Aβ peptide, and moderate inhibition of Aβ self-aggregation (34.9 %).[19, 20]

Interest on calcium channel blockers (CCBs) is based on the fact that calcium levels regulate neuronal plasticity underlying learning and memory and neuronal survival. Dysregulation of the intracellular calcium homeostasis in AD is thought to play a role in neuron degeneration and death.[21, 22]. Consequently, blocking the entrance of Ca^{2+} through L-type voltage-gated calcium channels is considered a valuable strategy to prevent neuronal damage in AD.[23, 24] Furthermore, CCBs have been shown to improve cerebrovascular perfusion and attenuate amyloid-β-induced neuronal decline and neurotoxicity, improve cell survival in the presence of Aβ *in vitro*, and exert neuroprotective effects in animal models.[25-27] These beneficial effects have been confirmed in clinical trials for the CCBs nimodipine and nilvadipine, which have reached phase III (ClinicalTrials.gov identifier NCT02017340).

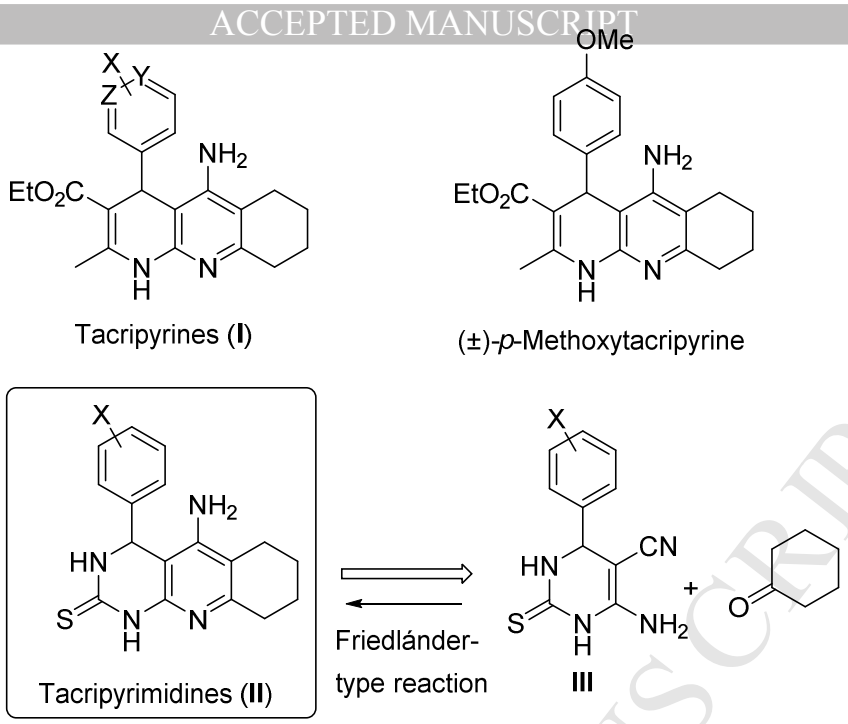


Figure 1. Structure of tacripyrines (**I**), (±)-*p*-methoxytacripyrine, and retrosynthetic analysis for the synthesis of tacripyrimidines (**II**).

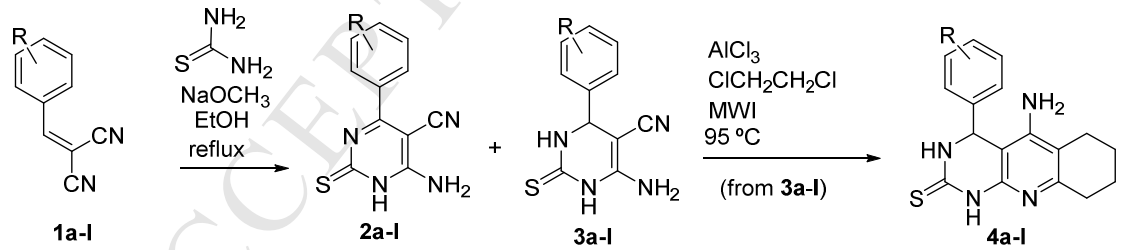
With these premises in mind and in the attempt of balancing the anticholinesterase and Ca-antagonism activities by increasing CCB activity, we have designed the related MTDs tacripyrimidines (**II**, Figure 1), by focusing again on tacrine scaffold as a template for cholinesterase (ChE) inhibition and on the well known capacity of 3,4-dihydropyrimidin-2(1*H*)-thiones (**III**, Figure 1) to act as efficient calcium channel blockers. [28, 29] Furthermore, very promisingly, dihydropyirimidine-thiones were recently shown to exert neuroprotective activity toward Aβ-induced toxicity in a yeast model for proteinopathies, likely by attenuating the metal-mediated toxicity of Aβ. [30]

Thus, the newly synthesized tacripyrimidines were investigated in terms of inhibitory activity toward both human ChE enzymes and calcium channels as main biological targets. The mode of interaction with human AChE (hAChE) of the most interesting derivatives was studied by a combination of *in vitro* and *in silico* approaches. Furthermore, because hepatotoxicity prevented tacrine from its clinical use [31], all tacripyrimidines were assayed on human hepatoma cells (HepG2) to assess their safety. Finally, the *in silico* calculation of

ADME parameters allowed assessing their ability to be absorbed after oral administration, to cross the blood brain barrier (BBB) and reach the central nervous system (CNS).

2.Results and Discussion

2.1 Compound synthesis. The synthesis of racemic tacripyrimidines **4a-l** (Scheme 1) has been carried out by a Friedländer-type reaction [32-34] between compounds **3a-l** [35] and cyclohexanone, under the usual experimental conditions (**Supporting Information**).[32] The key intermediates **3a-l** have been prepared here for the first time, albeit in modest yields due to the isolation of the corresponding unsaturated derivatives **2a-l**, by application of a Biginelli reaction from readily available arylidenemalonitriles **1a-l** and thiourea (**Supporting Information**).[36] As shown in Scheme 1, in addition to the parent compound **4a**, we have prepared tacripyrimidines bearing electron-donor [4'-Me (**4b**), 3'-Me (**4c**); 4'-OMe (**4d**), 3'-OMe (**4e**), 2'-OMe (**4f**); 3',4'-OCH_2O- (**4g**); 4'-NMe_2 (**4h**)] and electron-withdrawing [(4'-F (**4i**), (4'-Cl (**4j**), (4'-Br (**4k**); (4'-NO_2 (**4l**)] substituents at the aromatic ring at C4. All new compounds showed analytical and spectral data in full agreement with their proposed structures.



4a (X= H), **4b** (4'-Me), **4c** (3'-Me), **4d** (4'-OMe), **4e** (3'-OMe), **4f** (2'-OMe), **4g** (3',4'-OCH^2O-)
4h (4'-NMe_2), **4i** (4'-F), **4j** (4'-Cl), **4k** (4'-Br), **4l** (4'-NO_2)

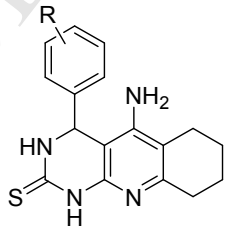
Scheme 1. Synthesis of tacripyrimidines **4a-l**.

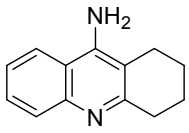
2.2 Biological Evaluation

2.2.1 Inhibition of Cholinesterases. With compounds **4a-l** in hand, we first addressed their hChE inhibition capacity, following Ellman's protocol [37].

As shown in table 1, most of tacripyrimidines **4a-l** are hAChEI with IC_{50} values ranging from 3.05 μM (**4e**) to 31.0 μM (**4i**), with the exception of tacripyrimidine **4k** (hAChE: IC_{50}= 0.0373 μM), the most selective and potent hAChEI within the series. On the basis of the inhibitory potencies, some structure–activity relationship (SAR) could be drawn. Tacripyrimidines bearing strong electron-donor (4'-NMe_2 for **4h**; 2'-OMe for **4f**) or electron-withdrawing (4'-NO_2 for **4l**) substituents, and moderate electron-donor (4'-Me for **4b**) or electron-withdrawing (4'-F for **4i**) substituents, regardless of their location at the aromatic ring, were the weakest hAChEIs. Among tacripyrimidines bearing a methoxy group, the most potent was compound **4e**, followed by **4d** and **4f**, position C3' being clearly preferred to C2' and C4'. This observation is also confirmed by the comparison of compound **4b** (4'-Me) and **4c** (3'-Me). Not surprisingly, the three most potent hAChEIs were the 5-amino-4-(3-bromophenyl)-3,4,6,7,8,9-hexahydropyrimido[4,5-b]quinoline-2(1*H*)-thione (**4k**), bearing a moderate electron-withdrawing substituent (Br) at C'3, followed by compound **4e** (3'-OMe), which was 81.8-fold less potent, and tacripyrimidine **4j** (C'4-Cl). Interestingly, compound **4k** was a 9.4-fold more potent inhibitor than tacrine.

Table 1. IC_{50} (μM) for the inhibition of hAChE and hBuChE by tacripyrimidines **4a-l** and tacrine.





Tacrine

Compd	R	hAChE[a] IC_{50} (μM) ± SEM	hBuChE[a] IC_{50} (μM) ± SEM	calcium intake blockade (%) ± SEM[b]
4a	H	10.8 ± 0.9	5.68 ± 0.38	10.58 ± 1.78$^{n/s}$
4b	4'-Me	23.8 ± 1.4	3.47 ± 0.15	14.69 ± 3.77**

4c	3'-Me	10.1 ± 1.1	2.65 ± 0.25	40.01 ± 4.45***
4d	4'-OMe	7.64 ± 0.43	1.75 ± 0.14	23.58 ± 2.31***
4e	3'-OMe	**3.05 ± 0.28**	**3.19 ± 0.11**	30.40 ± 2.61***
4f	2'-OMe	31.2 ± 1.5	11.7 ± 0.6	32.18 ± 2.92***
4g	3',4'-OCH_2O-	8.18 ± 0.97	37.5 ± 2.4	36.76 ± 3.57***
4h	4'-NMe_2	24.1 ± 3.1	154 ± 23	**59.01 ± 1.69*** **
4i	4'-F	31.0 ± 1.2	10.0 ± 0.7	23.31 ± 2.96***
4j	4'-Cl	5.28 ± 0.19	**0.372 ± 0.021**	38.00 ± 3.36***
4k	3'-Br	**0.0373 ± 0.0082**	1.27 ± 0.10	42.23 ± 3.85***
4l	3'-NO_2	29.8 ± 1.7	2.68 ± 0.15	**66.79 ± 2.41*** **
***p*-MT**[c]	-	0.105 ± 0.015	>100	32.75 ± 2.50
Tacrine	-	0.374 ± 0.053	0.0442 ± 0.0017	nd
Nimodipine	-	nd	nd	49.62 ± 1.24

[a] Results are expressed as the mean of at least two experiments in which each datum was obtained in triplicate; [b]All tacripyrimidines and nimodipine were assayed at 1 μM in three independent experiments each performed in quadruplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. n/s not significant, *P<0.05, **P<0.1, ***P<0.01 vs controls (vehicle). [c] ***p*-MT** stands for *p*-methoxytacripyrine (Figure 1); data from [19]. SEM stands for standard error of the mean; nd stands for not determined.

By comparing the inhibition rate of the compounds belonging to the tacripyrimidine series to reference compounds, it can be concluded that **4k**, the most potent AChEI in the tacripyrimidine series, is 10-fold more potent than tacrine in inhibiting hAChE, and it is also about 35 folds more potent than the best tacripyrine namely *p*-MT (Table 1).

Concerning inhibition of hBuChE, tacripyrimidines **4a-l** showed significant selectivity for hBuChE, with the exception of compounds **4g**, **4h**, and **4k** (which showed a reverse trend), with IC_{50} values spanning more than four orders of magnitude, i.e., varying from 0.372 μM (**4j**, the most potent hBuChEI within the series) to 154 μM (**4h**). Compound **4e** (3'-OMe) exerted a balanced inhibition of both ChEs, in the micromolar range.

Regarding the SAR for BuChE inhibition, quite similar trends were observed. Indeed, the most potent electron-donor substituents (4'NMe_2 in **4h**; 3',4'-OCH_2O- in **4g**, and 2'-OMe in **4f**) afforded the poorest hBuChEIs, C'3 being a preferred position for a better hBuChE

inhibition (compare compounds **4b** and **4c** for the Me group), although location at C'4 is also favored (compare compounds **4d**-**e** for the OMe motif). Compared with tacrine, compound **4j** was a 9.3-fold less potent hBuChEI.

It is interesting to note that tacripyrimidines showed a distinctly different behavior concerning BuChE inhibition in comparison with the previously developed tacripyridines, which were selective AChEIs with no significant activity toward hBuChE.[19] Increasing evidence has shown that inhibition of CNS BuChE activity may be beneficial for the treatment of moderate to severe forms of AD as highlighted by the increasing interest on BuChE as a therapeutic target in AD drug discovery [38] and by the design of BuChE-selective inhibitors [39, 40]. In fact, progressive elucidation of the role of BChE in AD brain has highlighted that, with the progression of the disease, the role played by AChE in the hydrolysis of the neurotransmitter acetylcholine (ACh) decreases. Conversely, BuChE levels and activity in certain regions of AD brain have been shown to increase [38]. Therefore, selective BuChE inhibitors (BuChEIs) could be more effective in patients with moderate to severe forms of this disease, although this tentative conclusion has not yet been clinically verified. Indeed, to date, no large-scale clinical trials of selective BuChEIs have been performed in patients with AD. Studies on mild to moderate AD patients, that is the population of patients enrolled in most clinical trials on ChEIs, is unlikely to be able to highlight the benefits of selective BuChE inhibition. In the light of these observations, the *p*-chloro tacripyrimidine **4j**, which was a 14.2-fold more potent hBuChEI, is worth further investigation. As a perfectly balanced micromolar ChE inhibitor, 3'-methoxytacripyrimidine **4e** is also worth to be considered.

2.2.2. Evaluation of the mode of ChE inhibition: *in vitro* and molecular modeling studies

To achieve a deeper understanding of the mode of interaction of our derivatives, tacripyrimidines **4e** and **4k,** the two most potent hAChEIs, were further investigated. Lineweaver-Burk plots for both compounds showed increasing slopes (lower v_{max}) but

unaltered intercept (unvaried K_m value) with increasing inhibitor concentration, indicating a non-competitive type of inhibition (Figure 2). This type of inhibition was further confirmed by data reprocessing according to the Dixon method (1/*v vs* [I]). The K'_i constant, i.e., the dissociation constant of the enzyme-substrate-inhibitor complex, has been calculated and found to be 3.88 ± 0.30 μM for **4e** and 0.147 ± 0.011 μM for **4k**, respectively. Importantly, the interaction of these compounds with the enzyme's PAS could be advantageous in the light of AChE's activity in promoting A*β* aggregation [6].

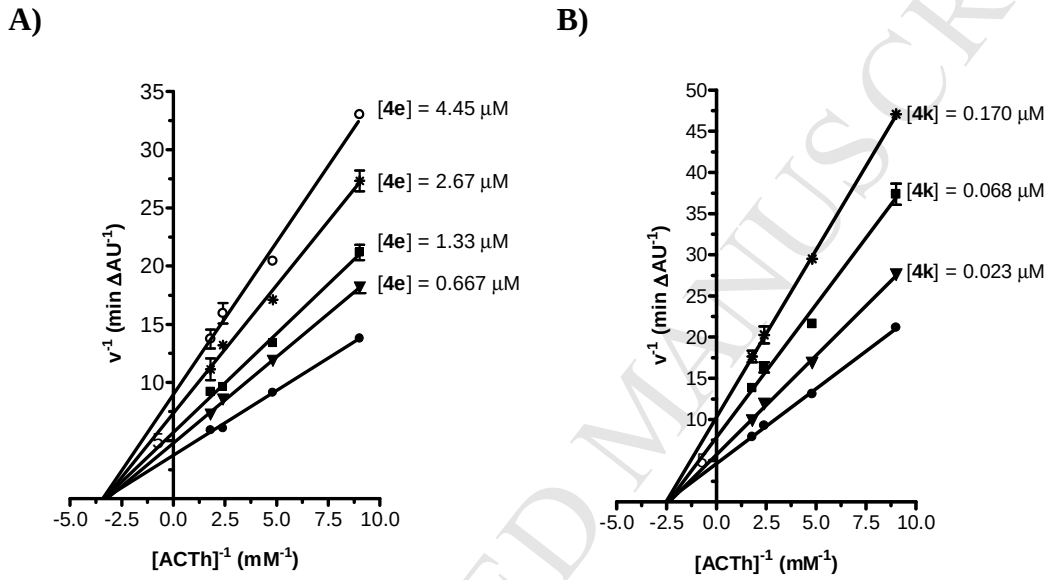


Figure 2. Kinetic study on the mechanism of hAChE inhibition by **4e** (A) and **4k** (B). Overlaid Lineweaver–Burk reciprocal plots of AChE initial velocity (v) at increasing substrate concentration in the absence and in the presence of inhibitor.

Based on the IC_{50} values and selectivity profile, we decided to look into the binding of selected tacripyrimidines **4k** and **4j**, the most potent and selective AChEI and BuChEI, respectively, on hAChE and hBuChE. The same analysis was also carried out for tacripyrimidine **4e**, the derivative showing no selectivity. Results are detailed in the **Supporting Information**. As initial study, the reported biological data refer to racemic mixtures, and the observed final results are a combination of the effects of both enantiomers.

Nevertheless, to obtain information about the mode of action, docking studies were performed on the (*R*)- and (*S*)-enantiomers of each selected compound. The ligand docking

studies were performed on the crystal structures of hAChE (PDB: 1B41) and of hBuChE (PDB: 1P0I) using Autodock Vina [41]. The docking procedure allows docking of ligands on the entire protein surface, without prior specification of the binding site. The recognition process between (*R*)- and (*S*)-enantiomers was investigated by flexible docking experiments. For both enzymes, flexible torsions in the ligands were assigned. In the case of hAChE, protein side chain flexibility was also incorporated, allowing the rearrangement of the side chains of eight residues, Trp286, Tyr124, Tyr337, Tyr72, Asp74, Thr75, Trp86 and Tyr341. The motion of these residues may increase the size of the gorge to facilitate the access of bulky ligand to the catalytic site.[20, 42]

In agreement with the experimentally confirmed non-competitive mechanism of action of (±)-**4k**, molecular modeling studies indicated that both (*R*)-**4k** and (*S*)-**4k** interact with residues at the PAS and not with those at CAS and highlighted the key interactions involved in the binding (Figures S1 and S2). On the other hand, concerning inhibition of hBuChE by **4k**, the best-ranked docking solutions revealed that (i) BuChE can effectively accommodate both enantiomers inside the active site gorge and (ii) both enantiomers have similar binding modes (Figures S4 and S5). The BuChE active site gorge is more voluminous than the AChE gorge. [43] Hence, it is not surprising that both enantiomers can reside in its large catalytic cavity.

Concerning the strong inhibitory activity of **4j** toward BuChE, best ranked docking poses revealed similar binding modes for both enantiomers inside the BuChE active site. In particular, both ligands bound BuChE very tightly, fully occupying its active site and establishing hydrogen bonds with the catalytic amino acids His438 and Ser198. Furthermore, the chlorine atom played a crucial role establishing a great network of halogen-bonding interactions with key amino acids located in the active gorge of BuChE (Figure S10). These interactions strongly stabilize the inhibitor-enzyme complex and are likely strongly contributing to the high inhibitory potency of **4j** toward hBuChE.

In general, the sets of interactions highlighted by docking studies on both ChEs (see Supporting Information for a detailed discussion) pointed out that the primary amino group and the halogen atoms are the features which mostly contribute to the inhibitory activities of compounds **4j** and **4k** toward hAChE and hBuChE. The position of the halogen atom also contributes to compound selectivity toward a specific ChE by establishing a network of interactions (see **Supporting Information** for a detailed discussion).

In order to explain the potency and selectivity of compounds **4k** and **4j**, ligand-complex interactions and binding free energies for their complexes with AChE and BuChE were analyzed. It is well known that halogen bonding plays a significant role in determining the stability of the ligand-enzyme complex and it is a very useful tool to enhance compounds affinities and specificities. Therefore, we focused on the halogen bonding interactions between the halogenated ligands **4k** and **4j** and the enzymes AChE and BuChE**.**

Bromine atom (compound **4k)** established a greater network of halogen-bonding interactions with AChE than with BuChE while chlorine atom (compound **4j)** established a denser network of halogen-bonding interactions with BuChE.

On the other hand, the analysis of the binding free energies for compounds **4j** and **4k** also suggested that the bromine atom can be responsible for the improved binding affinities of **4k** enantiomers within the AChE. Indeed, a decrease in binding affinity toward AChE was observed when the bromine atom was replaced with a chlorine ((*R*)-**4j** and (*S*)-**4j**, Table 2).

A comparison of the binding energies for the formation of the (*R*)- and (*S*)-**4k**-AChE complexes and of the (*R*)- and (*S*)-**4k**-BuChE complexes clearly showed a preference for AChE. The opposite trend is observed for compounds (*R*)-**4j** and (*S*)-**4j**, which showed a binding preference for BuChE. The same analysis was also carried out for tacripyrimidine **4e**, the derivative showing no selectivity. Results are detailed in the **Supporting Information** (Figures S12-18), and Table 2.

Table 2. Estimated binding free energies using AutoDock Vina for six ligand(**4k**,**4j**,**4e**)-ChEs complexes.

	hAChE			hBuChE		
Cmpd	**IC_{50} (μM) ± SEM**		Binding energy (kcal/mol)	**IC_{50} (μM) ± SEM**		Binding energy (kcal/mol)
4k	0.0373 ± 0.0082	(*R*)-**4k**	-11.1	1.27 ± 0.10	(*R*)-**4k**	-10.1
		(*S*)-**4k**	-10.5		(*S*)-**4k**	-10.3
4j	5.28 ± 0.19	(*R*)-**4j**	-9.3	0.372 ± 0.021	(*R*)-**4j**	-9.8
		(*S*)-**4j**	-9.5		(*S*)-**4j**	-10.3
4e	3.05 ± 0.28	(*R*)-**4e**	Mode I: -9.9 Mode II: -9.1	3.19 ± 0.11	(*R*)-**4e**	-9.3
		(*S*)-**4e**	-9.1		(*S*)-**4e**	-10.0

2.2.3. Inhibition of amyloid self-aggregation

Based on the weak inhibitory activity demonstrated by tacripyrines,[19] the three most interesting tacripyrimidines, namely derivatives **4e**, **4j** and **4k**, were tested for their antiaggregating activity against Aβ_{42} self-aggregation. To this purpose, the same 1/1 Aβ_{42}/inhibitor ratio used in the previous study was selected for screening and the thioflavin T (ThT) florescence assay was used to exert their potency in inhibiting Aβ fibril formation.[44, 45] The tested tacripyrimidines exhibited poor inhibitory potency, with a % inhibition of about 10%. These results confirm the dihydropyirimidine-thione scaffold is not optimal for inhibitory activity on amyloid aggregation, in agreement with recent studies by Tardiff *et al.* [30] who showed that the neuroprotective activity toward Aβ-induced toxicity exerted by some dihydropyirimidine-thiones was likely not ascribed to a direct action on amyloid fibrils but was mediated by their chelating properties.

2.2.4. Ca^{2+} Channel Blockade Activity of Tacripyrimidines

To verify the effectiveness of the rational design, we investigated the Ca^{2+} influx induced by K^+-depolarization in SH-SY5Y neuroblastoma cells, previously loaded with the fluorescent dye Fluo-4AM. Fluo-4-loaded cells were incubated in the presence of tacripyrimidines **4a-l** (1 μM) for 10 min and then stimulated with $KCl/CaCl_2$ solution in order to have a final concentration of K^+ and Ca^{2+} of 90 mM and 5 mM, respectively. Fluorescence emission intensity before stimulation and after stimulation was recorded at 535 nm (λ_{exc} = 485 nm). DMSO (0.01%) was used as a vehicle control. Nimodipine was used as a reference inhibitor, causing, at 1 μM, ~ 50% inhibition of K^+-evoked Ca^{2+} uptake.

All tacripyrimidines, except the unsubstituted derivative **4a**, significantly inhibited Ca^{2+} influx (Table 1) although to different extents. Interestingly, tacripyrimidines **4h** (bearing a dimethylamino substituent at position 4') and **4l** (bearing a nitro-group at position 3') were more potent CCBs than the reference drug nimodipine (59.01% vs 49.62% and 66.79% vs 49.62%, respectively). Furthermore, **4c** and **4k** showed a very good blockade activity (>40% inhibition) which was not significantly different (n.s., $P > 0.05$) from that of nimodipine. Finally, from a statistical point of view, the encountered slightly lower CCB activity of derivatives **4g** and **4e** was scarcely significantly different to that of nimodipine (*$P > 0.05$).

The calcium channel blockade activity of tacripyrimidines seemed unrelated to the electronic nature of the substituents at the aromatic ring. Indeed, the two most active compounds within this series, i.e., **4h** and **4l**, bore an electron-donor (4'-NMe_2) and an electron-withdrawing (3'-NO_2) group, respectively. If a homogeneous set of substitution is considered, as in the case of methoxy- or methyl-tacripyrimidines, the 4'-position seems the least favorable one (compare **4b** with **4c** and **4d** with **4e** and **4f**).

2.2.5. Evaluation of hepatotoxic effects on HepG2 cells.

Since, when dealing with tacrine derivatives, hepatotoxicity is one of the major issues to be addressed, the cytotoxicity of tacripyrimidines was evaluated *in vitro* using HepG2 cells.[46]

Cell viability at 24 h was determined by quantifying ATP as an indicator of metabolically active cells, using a luminescence-based assay (CellTiter-Glo® Assay, Promega). All tacripyrimidines were assayed at three different concentrations, from 10 to 300 μM. Tacrine was used as positive control. As shown in Table 3, in the selected assay conditions, tacrine did not significantly affected cell viability up to 100 μM, while at 300 μM a significant reduction of cell viability was detected. Among tacripyrimidines, only derivatives **4b** (4'-Me derivative) and **4j** (4'-Cl) showed higher hepatic cell toxicity than tacrine, while most tacripyrimidines were found to be similarly or slightly less toxic than tacrine. Quite promisingly, **4e** emerged as the safest tacripyrimidine, with no toxic effect on HepG2 cells even at the highest tested concentration. Although to a lower extent, **4l** also turned out to be safer than tacrine with a reduction of only 17% of the cell viability at the highest dose (300 μM).

Table 3. Toxicity against the human hepatoma cell line HepG2 exerted by tacripyrimidines **4a-l** and tacrine.

Cmpd	% of viability *vs* control ± SEM		
	10 μM	**100 μM**	**300 μM**
Tacrine	90.0 ± 0.5	91.1 ± 0.1	66.3 ± 3.7***
4a	105.0 ± 4.6	97.2± 2.0	68.2 ± 1.5**
4b	90.6 ± 0.7	62.0 ± 0.9***	45.2 ± 0.66***
4c	97.6 ± 0.6	82.5 ± 1.0**	68.1 ± 0.9**
4d	97.9 ± 1.1	100.2 ± 1.55	77.6 ± 2.1***
4e	**100.1 ± 0.7**	**102.4 ± 0.7**	**97.5 ± 0.5**
4f	98.1 ± 1.1	81.1 ± 0.8***	68.1 ± 0.5***
4g	103.1 ± 1.5	91.2 ± 1.45	64.1 ± 0.5**
4h	87.3 ± 0.5**	82.5 ± 0.2**	78.9 ±1.8**
4i	104.3 ± 1.7	79.1± 1.1***	72.5± 0.9***
4j	80.0 ± 0.5*	27.9 ± 0.38***	9.0 ± 5.6***
4k	103.2 ± 5.5	76.8 ± 58**	62.3 ± 2.3***

4l	**102.0 ± 6.4**	**96.5 ± 4.1**	**82.7 ± 0.8****

Cell viability is expressed as percentage over control (DMSO) as mean ± SEM of triplicate of three independent experiments. Comparison between tested compounds and control group was performed by one-way ANOVA. Statistical calculations were executed using SPSS version 19.0 (IBM Corp, Armonk, NY, USA) and considered significant when lower than 0.05. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

2.2.6. Prediction of ADME properties

Drugs for the treatment of a CNS pathologies such as AD must reach their site of action and must therefore meet more stringent requirements than peripheral drugs in terms of molecular weight, number of hydrogen bondings (HB) that the molecule can form both as donor and/or as acceptor, partition coefficient and bonds with rotational freedom. Particularly for CNS drugs, MW <450, HB donors <3, HB acceptors <7, QPlogPo/w <5, PSA (Polar Surface Area) <90, number of links with freedom of rotation <8 and hydrogen bonds <8 [47]. Furthermore, it is preferable that drugs are well absorbed orally. Computer-aided methods, nowadays, have become popular in the assessment of ADME properties and identification of good drug candidates.

In this light, several descriptors related to the ability of tacripyrimidine-thiones **4a-l** to be absorbed after oral administration and reach the CNS were calculated using QikProp software, version 3.8, (Schrodinger, LLC, New York, NY, 2013). Thus, about 45 physically significant descriptors and pharmacologically relevant properties of both enantiomers of tacripyrimidine-thiones were predicted and some of the important properties were analyzed. The results obtained (Table S1, Supporting Information) showed that the compounds under consideration satisfied Lipinski's 5-parameter rule,[48] indicating that these compounds could potentially be active at the CNS level. The hydrophilic/lipophilic relationship has a significant impact on many ADME properties. The compounds under study have lipophilic values within the limits. The partition coefficient (QPlogPo/w), a critical parameter for the estimation of membrane absorption, ranged between 2.295 and 3.439. According to Jorgensen’s rule-of-

three (ROT),[49, 50] a compound is likely to be orally available when at least two of the following criteria are satisfied: (a) the logarithm of predicted aqueous solubility, QPlogS>−5.7; (b) the predicted Caco-2 permeability, QPPcaco>22 nm/s and (c) the number of primary metabolites<7). All compounds fully satisfy Jorgensen's rule-of-three. The estimated percentage human oral absorption for the compounds is very high (82-100%). Concerning the penetration of blood-brain barrier, the most used parameter is the logBB. The logBB value of active drugs at the CNS level should be greater than -0.5; compounds with logBB value below -1.0 penetrate little into CNS. However, some commercial drugs active at the CNS level have logBB values <-1.0. The QPlogBB values for compounds **4a-l** have been calculated and are within the reported limits (-3<QPlogBB<1.2).

4. Conclusions

The development of new drug candidates for effective treatment of AD is a challenging task for medicinal chemists, and also a research area with potentially tremendous impact in society. In this scenario, the contribution of the academic research to the definition of an effective therapeutic intervention is destined to increase as a consequence of the recent cut of AD focused research programs by some pharmaceutical companies.[8] Having this in mind, we have synthesized and investigated new 5-amino-4-aryl-3,4,6,7,8,9-hexahydropyrimido[4,5-*b*]quinoline-2(1*H*)-thione **4a-l**, as MTDLs able to modulate both cholinesterase activity and calcium influx mediated by voltage-dependent calcium channels. Indeed, *in vitro* and *in silico* studies showed that activity toward the two selected targets is modulated by the substituent attached to the aromatic ring at position C4. Derivatives bearing halogens (Br, Cl) at C3' and/or C4' position showed the highest inhibitory potencies toward hChEs while the introduction of a dimethylamino group at position 4' or nitro group at position 3' afforded the best calcium channel blockers, with potencies higher than that of the reference CCB drug nimodipine. Considering the overall biological profile of tacripyrimidines, including predicted ADME parameters and hepatotoxicity, tacripyrimidine

4e emerged as the first well balanced (low micromolar inhibitory activity) inhibitor of ChEs (50% inhibition at ~3 μM on both ChEs) and calcium channel (30% inhibition at 1 μM), endowed with no significant hepatotoxicity toward HepG2 cells up to 300 μM and excellent predicted oral absorption and BBB permeability. Indeed the few previous attempts to get MTDLs with AChE/BuChE and calcium channel inhibitory activities did not succeed in having these activities fully balanced [19,20,51]. Worth to further note, experimental evidence from the use of BuChE selective compounds and non selective inhibitor of both AChE and BuChE indicates potential therapeutic benefits for the treatment of AD and related dementias when both enzymes are inhibited.[52]

Not less importantly, in the light of the role of BuChE as prevalent ACh degradating enzyme in moderate-to-advanced forms of AD and considering the potential role of BuChE in the etiology and progression of AD, the 3'-nitrotacripyrimidine **4l,** which acts as moderately selective micromolar hBuChE inhibitor (AChE/BuChE = 10, $IC_{50(BuChE)}$ = 2.68 μM) and potent CCB with a significantly higher activity than nimodipine, can also be considered as a promising candidate for further development. To note, **4l**, although less safe than **4e,** resulted significantly less hepatotoxic than tacrine, exerting a limited toxicity only at the highest tested concentration (300 μM). In our opinion the best performing tacripyrimidines hold a promising activity and safety profile and are worth of further investigation and development.

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Declarations of interest: None.

Supporting Information

Experimental part including chemistry, NMR spectra and in vitro assays, molecular modeling studies on derivatives **4k**, **4j** and **4e**, calculated physicochemical properties for tacripyrimidines **4a-4l**. This material is available free of charge via Internet at http://

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Highlights

- Twelve tacripyrimidines were designed and synthesized
- Tacripyrimidines acted as modest-to-good cholinesterase inhibitors
- Most tacrypirimidines also acted as moderate-to-potent calcium channel blockers
- **4e** emerged as a well balanced cholinesterase inhibitor and calcium channel blocker
- **4e** was not hepatotoxic and showed a good predicted oral absorption and BBB permeability